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(54) Title: METAL-BASED FORMULATIONS WITH HIGH MICROBICIDAL EFFICIENCY VALUABLE FOR DISINFECTION AND STERILIZATION

(57) Abstract

Transition metals such as copper and iron, alone or in combination with each other or other transition metals, in aqueous solution are able to inactivate bacteria fungi, and viruses with an efficiency similar to that of substances usually recommended for disinfection and sterilization. Other transition metals can be used as disinfectant and sterilizing agents. The inactivating efficiency of such metals can be greatly enhanced by the addition of hydrogen peroxide, other peroxides, or pero-generating agents. The microbicidal potency of such mixtures can be increased even further in a ternary system composed of metal(s), peroxide, and a chelator, such as nitrilotriacetic acid (NTA). Different formulations of mixtures of metals and peroxide with or without NTA are provided. These are capable of inactivating viruses, bacteria, and fungi with an efficiency higher than that exhibited by substances currently in use. Also provided are methods for inactivating microorganisms for vaccine development, sterilizing or disinfecting medical devices and fluids, treating infectious disease, and assaying disinfectant, sterilizing, and antimicrobial substances and complex mixtures for microbicidal efficiency.

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METAL-BASED FORMULATIONS WITH HIGH MICROBICIDAL EFFICIENCY VALUABLE FOR DISINFECTION AND STERILIZATION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to chemical inactivation of microorganisms, particularly bacteria and viruses. Effective inactivation of infectious microorganisms is of importance in the sterilization and disinfection of reusable and some disposable medical devices, as well as for implantable grafts and prostheses. The present invention addresses current problems in sterilization and disinfection by describing metal solutions, metal/peroxide mixtures, and metal-peroxide-chelator systems of high efficiency in inactivating viruses and bacteria. This invention is also relevant to vaccine development, where appropriate inactivation is required to preserve antigenicity of microorganisms. Inactivating pathogenic microorganisms via the use of the efficient formulations described herein can also form the basis for therapeutic treatment to cure or control infection.

Description of Related Art

Inactivation of microorganisms is used to disinfect and sterilize medical devices, in the therapy of infectious diseases, and to develop attenuated vaccines. In particular, liquid sterilization and disinfection are carried out extensively in hospitals as well as by the biomedical and pharmaceutical industries.

The current AIDS epidemic caused by Human Immunodeficiency Virus (HIV) has brought new interest to infection transmission by a wide range of medical devices used in the clinical environment. The significant increase in individuals infected with HIV has augmented the risk of patient to patient transmission of AIDS by medical devices [Hanson and Collins, Thorax, 44:778 (1989); Weller, Gut, 29:1134 (1988); Bond, JAMA, 257:843 (1987)]. Also, infection by opportunistic microorganisms in AIDS and other immune-suppressed patients is often fatal. These facts indicate the for protection of the

noninfected population, as well as for AIDS patients, disinfection of medical equipment before use must be considered of utmost importance.

The finding of diverse infectious agents in medical devices, particularly of HIV in fiberoptic bronchoscopes, has led to the notion that the primary cause of nosocomial infection is the transmission of infectious agents by multiple uses of inappropriately sterilized medical devices [Favero, in Manual of Clinical Microbiology, Am. Soc. Microbiol., Washington, D.C. (1985), p. 129].

The devastating consequences of HIV infection and the larger number of symptomless infected individuals dictate the need for adequate procedures for disinfection and sterilization for medical devices.

The true nature of infectious complications due to contaminated medical devices is difficult to determine and is generally inaccurate owing to under-reporting and to the long incubation times of several pathogens, such as HIV and the agent causing Creutzfeldt Jakob disease. Although often referred to as a fragile virus, HIV can remain infectious at room temperature for up to three days if dried, and for eight days in suspension [Resnick et al, JAMA, 255:1887 (1986)].

Sampling of a series of bronchoscopes used on patients with AIDS showed contamination with respiratory tract commensals, Candida albicans, hepatitis B virus, and in all cases HIV [Hanson and Collins, Thorax, 44:778 (1989)]. Persistent contamination of bronchoscopes with Serratia and Pseudomonas has been described [Pappas et al, Am. Rev. Respir. Dis., 127:391 (1983); Webb et al., Chest, 68:703 (1975); Siegman et al, J. Hosp. Infect., 6:218 (1985)]. Mycobacterium chelonei, tuberculosis, and avium have also been transmitted by contaminated endoscopes [Leers, Can. Med. Ass. J., 123:275 (1980), Dawson et al, Am. Rev. Resp. Sis., 126:1095 (1982)]. The outbreak of infections with Psuedomonas and Bacillus

species related to fiberoptic bronchoscopes has been reported [Hussain, Chest, 74:483 (1978); Goldstein and Abrutyn, J. Hosp. Infection, 6:194 (1985)].

Incidences of 6% for pneumonia, 30% for bacteriemia, and 46% for fever after bronchoscopies have been shown [Pereira et al, Am. Rev. Resp. Dis., 112:59 (1975); Burman, J. Thorac. Cardiovasc. Surg., 40:635 (1960)]. Although the contamination of bronchoscopes seems to be the best documented, several other medical devices have been related to transmission of disease. Transmission of hepatitis by contaminated medical devices has long been a concern of surgeons and clinicians. Bacterial contamination inside a spirometer after one week use has been measured at 10^8 organisms/ml [Houston et al, Breath, 12:10 (1981)]. The risk of transmitting infectious microorganisms by some models of ventilators and respirators has also been suspected.

The well documented transmission of Creutzfeldt Jakob disease (CJD) (a form of progressive dementia) to patients receiving contaminated duramater from cadavers led the American Association of Tissue Banks and the Food and Drug Administration to conclude that current disinfection procedures are not sufficient to completely inactivate CJD [Am. Ass. Tissue Banks News, 10:1 (1987); JAMA, 257:1289 (1987)]. Duramater is used primarily in neurosurgery, and also in orthopedic, otologic, dental, urologic, gynecologic, and cardiac procedures, calling for proper disinfectants in all these specialties.

The reusable devices which come in contact with blood and body tissues, and which can thus be potential vectors for the transmission of HIV and other microorganisms are: surgical instruments, including those used in dentistry, catheters, implants, hemodialyzers, fiberoptic instrumentation for exploratory purposes, endoscopes for biopsy, and fiberoptic endoscopes with their accessories, used in diagnosis and therapy [Weller, Gut, 29:1134 (1988); Raufman and

Strauss, Gastroenterology Clin., 17:495 (1988); Bond, JAMA, 257:843 (1987); Favero, in "Manual of Clinical Microbiology," 129, Am. Soc. Microbiol., Washington, D.C. (1985)]. In addition, graft prostheses such as duramater, skin, corneas, and heart valves of human origin have been implicated in the transmission of disease, including AIDS [Am. Ass. Tissue Banks News, 10:1 (1987); Manuelidis et al, New Engl. J. Med., 296 (1977)].

A number of chemical sterilizing agents have been used. Based upon the inactivation of Hepatitis virus, The American Society for Microbiology [Gorschel et al, Laboratory Safety, Principles and Practices, p. 56, Am. Soc. for Microbiology, Washington, D.C. (1986)] has suggested the use of the following solutions:

For sterilization, (a) Glutaraldehyde, aqueous 2%; (b) Hydrogen peroxide, stabilized 6 to 10%; and (c) Formaldehyde, aqueous 8 to 12%.

For disinfection, (a) Glutaraldehyde, aqueous at variable concentration; (b) Hydrogen peroxide, stabilized at 6 to 10%; (c) Formaldehyde, aqueous between 3 to 8%; (d) Iodophores containing either 30 to 50 mg/L of free iodine, or 7 to 150 mg/L of available iodine; and (e) Chlorine compounds at 50 to 500 mg/L of free available chlorine.

The choice of disinfectant for use with medical instruments is greatly limited by its corrosive properties. Even moderate concentrations of phenolics, hypochlorite, and iodophores damage many delicate medical devices.

Mutagenicity and carcinogenicity in experimental animals has been reported for formaldehyde. An association between the increased risk of spontaneous abortion and exposure to formaldehyde has been indicated [Hemminki et al, Am. J. Industrial Med., 4:293 (1983); Shumlina, Prof. Gig. Zabol., 19:18 (1975)]. However, Gigasept (a formaldehyde based reactive) is used as an

alternative by personnel sensitized to glutaraldehyde (see below).

Ethylene oxide has also been used for sterilization, but reported increased risk of mutagenesis, carcinogenesis, and spontaneous abortion, as well as the demonstrated decrease in the duration of function of tissue homografts, has prevented its widespread acceptance [Wallace, Am. J. Cardiol., 35:866 (1975)].

Transmission of Hepatitis B virus to chimpanzees was shown to be prevented by treating the virus with 2% alkaline glutaraldehyde for 5 to 10 minutes [Bond et al, J. Clin. Microbiol., 18:535 (1983); Kobayashi et al, J. Clin. Microbiol., 20:214 (1984)].

Based upon these and other studies, the "Working Party" of the British Society of Gastroenterology recommended that contaminated bronchoscopes should be disinfected for 4 minutes in 2% alkaline glutaraldehyde after cleaning [Weller, Gut, 29:1134 (1988)].

Most of the available disinfection data are related to bronchoscopes; disinfection practices for other medical devices tend to follow those used by bronchoscopy units.

Interestingly, transition metals alone, such as Ag, have been used to disinfect wounds contaminated with bacteria, and to treat bone infections [Becker et al, Journal of Bone and Joint Surgery, 60A: 871-81 (1978); Spadara, Antimicrobial Agents and Chemotherapy, 6:637-42 (1974); Berger, Antimicrobial Agents and Chemotherapy, 10:856-60 (1976)].

Transition metals are also known to exhibit antitumor effects [Spadara, Proceedings of the Third Annual Biomaterials meeting (1977)].

Although glutaraldehyde is the disinfectant of choice, there remain serious problems associated with its use. A survey of 43 endoscopy units in the United Kingdom disclosed that 37% of their units had problems due to sensitization of the staff [Axon et al, Lancet, i,

1983 (1981)]. Most affected are those with greater exposure, i.e., the most experienced endoscopy assistants and those who work in units with the fewest staff. These are the very people units can least afford to lose. As the problem is common to all aldehydes, changing the formulation of the disinfectant brings at best only temporary relief.

The inactivation of HIV by alkaline glutaraldehyde is affected by the presence of protein. HIV in serum remains infectious for over 15 minutes in 1% glutaraldehyde [Hanson et al, Brit. Med. J., 298:862 (1989)]. This is important since reuse of glutaraldehyde after about 20 bronchoscopic procedures reduces its active concentration to about 1%. [Hanson and Collins, Thorax, 44:788 (1989)].

In spite of its drawbacks on efficacy and staff sensitization, exposure of medical devices to 2% glutaraldehyde for 4 to 20 minutes is now the disinfection method of choice, and is used in medical units throughout the world [Church et al, Thorax, 43:849P (1988); Hanson and Collins, Thorax, 44:778 (1989)]. There is simply no better alternative.

The diversity of infection control precautions taken by physicians, together with the accumulated data indicating nosocomial infection after use of medical devices, suggests that current disinfection practices are clearly unsatisfactory [Church et al, Thorax, 43:849P (1988)].

A better sterilizing agent, harmless to humans and medical devices, and capable of quickly and efficiently inactivating microorganisms, in particular viruses, would be a major contribution to the quality of health care.

SUMMARY OF THE INVENTION

As disclosed herein, transition metals such as copper and iron are more efficient biocidal agents than formaldehyde, chlorine or peroxide. The microbicidal

effect of metals is further enhanced by peroxide. The potentiation by peroxide is concentration dependent, and increased throughout the range described herein. For example, a mixture of copper or iron and peroxide in a 1:100 ratio is 50 and 5 times more efficient than glutaraldehyde in inactivating viruses and bacteria, respectively. Nitrilotriacetic acid (NTA) potentiates approximately seven times further the virucidal potency of a metal peroxide mixture.

Accordingly, it is an object of the present invention to provide a disinfectant or sterilizing composition comprising an aqueous solution of a salt of a transition metal or metals, such as copper or iron, either alone or in combination with a peroxide, a peroxide and a chelating agent, such as nitrilotriacetic acid, or in combination with a chelating agent alone.

It is another object of the present invention to provide a method for inactivating viruses, bacteria, or fungi, comprising treating said viruses, bacteria or fungi with said disinfectant or sterilizing composition.

A further object of the present invention is to provide a method for disinfecting or sterilizing laboratory equipment, such as benchtops, medical devices, prosthetic devices, homo- and heterografts, synthetic grafts, implants, fluids such as blood and plasma, and similar materials or devices for use on or in the human body by treating the same with said disinfectant or sterilizing composition.

A still further object of the present invention is to provide a method for inactivating microorganisms to facilitate their use in the production of vaccines comprising treating said microorganisms with said disinfectant or sterilizing composition.

Yet a further object of the present invention is to provide a method for treating infectious diseases in a mammal, including humans, as well as a method for disinfecting wounds and for treating bone infections

therein, comprising administering to an afflicted subject an antimicrobial effective amount of said composition as a pharmaceutical composition.

Yet another object of the present invention is to provide a method for treating tumors in a mammal, including humans, comprising administering to an afflicted subject an antitumor effective amount of said composition.

Still a further object of the present invention is to provide methods for assessing the microbicidal efficiency of disinfectant, sterilizing, and antimicrobial substances, and complex mixtures.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawings provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed descriptions taken in conjunction with the accompanying drawings, all of which are given by way of illustration only, and are not limitative of the present invention, in which:

FIG.1 shows the comparative virucidal effect of substances recommended for liquid sterilization and disinfection: Glutaraldehyde (Gl), Chlorine (Cl), Hydrogen peroxide (Px), and Formaldehyde (Fm). Virus remaining infectious after 30 min. incubation at 21°C in the presence of varying concentrations of active substance is plotted, and the concentration required to inactivate half the virus load is indicated.

FIG. 2 illustrates the virucidal property of different concentrations of copper (Cu, as copper chloride) and iron (Fe, as iron chloride) as compared with glutaraldehyde (Gl). The effect of sodium chloride (NaCl) is included to show that chloride ions do not contribute to the virucidal effect observed with the metallic salts.

FIG. 3 demonstrates the cooperative effect on virus inactivation produced by peroxide and copper. The virucidal capacities of peroxide alone (Px) and copper alone (Cu) are compared with an equimolecular mixture of both substances (Mix 1).

FIG. 4 illustrates the potentiating effect of different proportions of peroxide on the virucidal activity of copper. The virus was treated for 30 min. at 21°C in the presence of 3.17 mg/L of copper and a peroxide concentration ranging from 1.7 to 170 mg/L (upper horizontal axis). This range corresponds to one molecule of peroxide per molecule of copper, to 100 peroxide molecules per copper molecule (lower horizontal axis).

FIG. 5 shows the higher virucidal activity of a mixture of 100 parts of hydrogen peroxide per part of copper (Mix 100) when compared with glutaraldehyde (Gl). The graph represents infectious virus remaining after 30 min. incubation at 21°C (Y axis) as a function of either the copper concentration in the mixture or the concentration of glutaraldehyde (X axis).

FIG. 6 shows the higher bactericidal activity of a mixture of 100 parts of hydrogen peroxide per part of copper (Mix 100) when compared with glutaraldehyde (Gl). The number of bacterial colonies remaining after 30 min. incubation at 21°C with either Mix 100 or Gl is plotted as a function of either copper concentration in the mixture or glutaraldehyde concentration.

FIG. 7 depicts the results of three independent experiments performed to compare the rate of virus

inactivation at room temperature between a mixture of 1 mg/L of copper (as copper chloride) and 100 mg/L of hydrogen peroxide, versus 1 mg/L glutaraldehyde. The effect of incubation in the presence of sodium chloride (NaCl) is included as a control to demonstrate virus stability during the time interval studied.

FIG. 8 shows the potentiating virucidal effect of NTA over ferric ions and peroxide corresponding to 1 mg/L each. Virus in 0.9% serum was incubated during 30 min. at 21°C in the absence (0 g/L) or in the presence of NTA at various concentrations. Iron was chelated to NTA at pH 7.8 prior to incubation with virus and peroxide.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention, as modifications and variations in the embodiments herein discussed may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

I. INTRODUCTION

The capacity of different substances to inactivate microorganisms is used to disinfect and sterilize medical devices, to treat infectious diseases, and to develop attenuated vaccines.

The model described here for assessing virucidal activity was developed using the attenuated strain clone 3 of Junin virus (JV), which has been previously used as an experimental vaccine [Ruggiero et al, J. Med. Virol., 7:227 (1981)]. JV is classified within the Arenavirus family. Like HIV, JV is an enveloped virus whose RNA genome is surrounded by protein [Bishop, in Virology, p. 1231, Raven Press (1990)]. The attenuated strain of JV used in this work was chosen because it is harmless to

humans, and is readily available. Other viruses could have been used as well without departing from the technical considerations described herein.

The main advantages of the virucidal assay described here are:

(1) It can compare substances of very different activities. By comparing the doses that inactivate fifty percent of microorganisms (ID_{50}), the assay can be performed with substances at concentrations ranging from 0.4 μg/L to 10 g/L. This range can be extended even more if necessary.

(2) It uses minimal amounts of sample, so that it can eventually be used to test the inactivation of microorganisms in the small volumes available from clinical specimens. The total volume of the assay was reduced to 28 μl.

The present invention discloses solutions of transition metals alone, a mixture of such metals and peroxide, a mixture of transition metals and peroxide with a chelating agent, and solutions of transition metals with a chelating agent, having a very high efficiency for inactivating cells of various types, including tumor cells and microorganisms such as viruses, bacteria, and fungi. The microbicidal potency of metal-peroxide mixtures was enhanced by the addition of the chelator NTA. The high virucidal efficacy of such metal-based formulations was maintained in the presence of serum.

The present invention focuses on the properties of copper and iron because of the relatively low risk they pose to human health. No sensitization is known to develop after exposure or contact to either of these metals. This is a major advantage over current liquid disinfectants and sterilizing agents. Treatment of medical devices with transition metals such as copper or iron in the form of chloride, sulfate, or other salts should be well tolerated.

Homografts have been subjected to various methods of sterilization, including the use of glutaraldehyde and formaldehyde. It has been concluded that all current methods are insufficient to completely inactivate pathogens such as CJD [Am. Ass. Tissue Banks News, 10:1 (1987); JAMA, 257:1289 (1987)]. The high efficiency of a mixture of a metal and peroxide affords a better safety margin for the inactivation of microorganisms present in homo- as well as hetero-graft material. The higher activity of metals in the presence of peroxides, and further enhancement by NTA, also allows for gentler treatment of tissues, promoting longer functional life.

Many of the substances used for sterilization and disinfection have also been used for inactivating microorganisms in the development of attenuated vaccines. The loss of antigenic determinants by treatment with inactivating agents such as formaldehyde has made the development of vaccines to some diseases difficult [Videla et al, J. Med. Virology, 29:215 (1989)]. Metals, either alone, with peroxide, in a ternary mixture with peroxide and NTA, or with NTA alone, offer an alternative for attenuation of microorganisms for vaccine development.

Copper and iron are present in the human body under normal physiological conditions. The concentration of copper and iron in human sera, 1.1 mg/L and 1.9 mg/L, respectively, [Scientific Tables Geigy, p. 585 (1965)] is within the range of concentrations described herein. Peroxide is released at a rate of 0.17 to 0.34 ng per million cells in a 5 min. period after activation of either macrophages or polymorphonuclear leukocytes present in human blood [Nathan and Root, J. Exp. Med., 146: 1648 (1977)].

A. Materials

Vero cells were obtained from the America Type Culture Collection (Rockville, Md). Eagle's minimal

essential media (EMEM) and fetal calf serum (FCS) were purchased from either Gibco (Grand Island, NY) or Advanced Biotechnologies (Columbia, MD). The original source of the attenuated strain of Junin virus was a generous gift from Col. C.J. Peters (USAMRIID, Ft. Detrick, MD). Strain NM514 of E. coli was purchased from Amersham (Arlington Heights, IL), and cultivated in the laboratory.

Copper chloride ($CuCl_2 \cdot 2H_2O$), nitrilotriacetic acid (disodium salt), and formaldehyde 37% were purchased from Sigma Chemical Co. (St. Louis, MO). Iron chloride ($FeCl_3 \cdot 6H_2O$) and hydrogen peroxide 30 % (stabilized) were obtained from Mallinkrodt Inc. (Paris, KY). Glutaraldehyde 8% in sealed glass ampules was purchased from Ladd Research Industries (Burlington, VT), and diluted daily before experiments. Cidex (Surgikos, Johnson and Johnson) was generously supplied by Mr. Z. Glazer. CHLOROX was used as a source of 5.25% sodium hypochlorite.

L-broth media and 85 mm LB-agar Lennox plates used in the bactericidal assay were purchased from Gibco/BRL (Grand Island, NY). The 6 well or 24 well plastic tissue culture plates used for titration were supplied by Corning Glass Co. (Corning, NY).

B. Cell cultures and virus preparation

Vero cells were grown in 175 cm^2 plastic tissue culture flasks (Corning Glass Co., Corning, NY) in EMEM, pH 7.3, supplemented with 5% inactivated FCS at 37°C under a CO_2 atmosphere. When the cell sheet reached about 80% confluence, the culture medium was removed, and the cell sheet washed once with phosphate buffered saline (PBS), pH 7.1, before infection.

Cell monolayers were infected with Junin virus for 1 hr at 37°C; 30 ml of EMEM were added and incubated at 37°C in a CO_2 atmosphere. Viruses were harvested 5 days after infection.

C. Preparation of solutions

Metal salts were typically dissolved at 1 g/L in 1 ml distilled water (in some cases acidified with 10 µl 2 N hydrochloric acid), and sterilized by filtration through 0.22 µm membrane filter units (Millex GV, Millipore, Bedford, MA).

Inactivating agents were serially diluted 1:10 in sterile distilled water at neutral pH, and experiments performed within 3 hr thereafter. New dilutions from the stock solution were made before each experiment.

D. Assessment of virucidal activity

Junin virus at a concentration of about 10^6 PFU/ml in EMEM containing 5% FCS was diluted 1:4 in PBS, pH 7.4, before adding the inactivating agent at various concentrations. The reaction volume was 28 µl, and incubation was carried out in sterile conical 1.5 ml Eppendorf tubes for 30 minutes at room temperature (21°C). After 30 minutes incubation, the reaction mixture was diluted to 1 ml with ice-cold EMEM-5% FCS, and put on ice. Remaining viruses in each sample were titrated immediately by overlaying 0.2 ml over 80% confluent Vero cells in 35 mm diameter 6 well tissue culture plates. The adsorption period was 1 hr at 37°C, after which the inoculum was removed and cells washed with 2 ml of PBS per well. To each well, 2 ml of 0.5% agar in EMEM-5% FCS were carefully overlaid, and after solidifying, 2 ml EMEM-5% FCS were added. Cells were incubated at 37°C for 5 days, after which they were stained for 4 hr with 2 ml of 0.1% crystal violet/4% formaldehyde. Between 200 and 250 virus plaques per well were typically obtained in untreated controls.

E. Determination of inactivation kinetics

Kinetic experiments were carried out in 1.5 ml plastic tubes by adding 50 µl of virus (at about 10^6 PFU/ml) in EMEM-5% FBS, and 115 µl of either water, PBS,

pH 7.1, or EMEM (pH 7.3)-5% FCS. The substance under test (115 μl) at the proper concentration was added, and timing began. A 28 μl aliquot from each sample was immediately withdrawn, and was assumed to correspond to time zero of incubation. At five additional preestablished time intervals, 28 μl aliquots were withdrawn from each sample. All aliquots were immediately diluted to 1 ml with ice cold EMEM-5% FCS after withdrawal, and kept on ice until the last time point was taken. Infectious viruses in 0.2 ml of each time point aliquot were titrated over Vero cell monolayers in 6 well plastic culture plates as described above.

F. Assessment of bactericidal activity

An individual colony was picked from a stock plate of E. coli, and inoculated into 10 ml L-broth media with 0.4% maltose. After incubation at 37°C overnight with shaking (200 rpm), 1 ml of culture was added to 50 ml prewarmed L-broth-0.4% maltose. Cultures were vigorously shaken at 37°C until an OD_{600} of 0.75 was reached (approximately 3.7×10^8 cells/ml). Five μl of bacteria in the logarithmic phase of growth were diluted 1:4 in PBS, and 8 μl of the substances to be tested at the desired concentrations were added in sterile plastic Eppendorf tubes. After 30 min. incubation at 21°C, samples were diluted to 1 ml with cold LB broth-0.4% maltose, diluted again 1:10, and 10 μl inoculum plated onto 85 mm agar Petri dishes. Typically, about 300 colonies per dish were obtained for untreated controls.

It is pointed out that copper and iron are given by way of exemplification only as the list of transition metals capable of use for disinfection and sterilization is neither exhaustive nor limiting. These include metals in Groups II-IV, for example. Hydrogen peroxide was used to gather the pertinent data herein because it is relatively inexpensive and safe to use. However, other

peroxides or pero-generating agents can be used for enhancing the microbicidal effect of metals without departing from the spirit or scope of the invention. NTA efficiently forms chelate complexes with many metals, and is given here as an example.

Although NTA produced the greatest enhancement of virucidal activity of metal:peroxide mixtures, other metal chelators, such as EDTA, EGTA, and bathocuproine, can be employed.

II. RESULTS

A. Comparison among currently employed disinfectants

Several liquid disinfectant and sterilizing substances are currently recommended, based mainly upon inactivation of Hepatitis Viruses [Groschel et al, Laboratory Safety, Principles and Practices, p. 56, Am. Soc. Microbiology, Washington, DC (1986)]. Aqueous glutaraldehyde 2%, stabilized hydrogen peroxide 6 to 10%, and aqueous formaldehyde 8 to 10% are recommended for sterilization. Variable concentrations of glutaraldehyde, formaldehyde 3 to 8%, and peroxide 6 to 10% are considered to have high disinfectant activity, while iodophors (30 to 50 mg/L free iodine or 70/150 mg/L available iodine) and chlorine compounds (50 to 500 mg/L free available chlorine) are considered to have intermediate disinfecting activity.

It was desirable to assess the inactivating activity of the different recommended liquid sterilizing agents in the virus assay described above. The comparative study shown in Figure 1 indicates that glutaraldehyde at neutral pH is the most active virucidal substance. Indistinguishable results were obtained for alkaline glutaraldehyde activated by adding bicarbonate (CIDEX) as compared with glutaraldehyde brought to neutral pH by buffered media immediately before assay.

The relative doses of different disinfectants needed to reduce 50% of viral infectivity (Inactivating Doses

ID_{50}) are also indicated in the figure. It is remarkable that agents recommended as sterilizants such as peroxide and formaldehyde exhibited 20 to 100 times less virucidal power, respectively, than glutaraldehyde (Table I). This finding indicates that glutaraldehyde is the most active virucidal agent in use, and that any newly proposed substance should be compared with it.

TABLE I

VIRUCIDAL EFFICIENCY OF SUBSTANCES USED IN STERILIZATION AND DISINFECTION AS COMPARED WITH METAL-BASED MIXTURES

Substance	Mixture[a]	ID_{50} (mg/L)[b]	Virucidal power
copper[d]	100:1	0.07 0.03	54
	1:1	6.1 3.3	0.6
	alone	16 10	
iron[d]	100:1	0.6	4.9
	1:1	4.1	0.7
	alone	24	0.1
glutaraldehyde		1.8 3.4 1.7 5.0 1.5	1.0
peroxide		110 160	0.02
formaldehyde		550	0.005
chlorine[e]		150	0.02

a: Mixture is referred to as mg of hydrogen peroxide per mg of substance.

b: In all experiments, 0.9% fetal calf serum was present during virus incubation.

c: Virucidal power is compared to the ID_{50} of glutaraldehyde, and calculated as ID_{50} for the substance alone or in a mixture, divided by the ID_{50} (average) of glutaraldehyde.

d: The chloride salts of Copper (II) and Iron (III) were used.

e: As sodium hypochlorite.

B. Virucidal effect of metals

Figure 2 shows the virucidal effect of iron (FeIII) and copper (CuII) in comparison with that of glutaraldehyde. Based upon ID_{50} values, copper and iron are approximately 5 and 10 times less active, respectively, than glutaraldehyde (see Table I). The ID_{50} of copper and iron compare favorably to the ID_{50} observed for recommended disinfectants such as formaldehyde, peroxide and chlorine (see Figure 1 and Table I). The survival of infectious viruses after incubation with saline (sodium chloride) proves that the virucidal effect of the copper and iron salts is not due to the chlorine but to the metal.

The results shown here were obtained with copper (II) and iron (III). Metals in other oxidation states, such as copper (I) and iron (II), also exhibit inactivating properties (data not shown).

C. Enhancement of the virucidal effect of metals by peroxide

In an attempt to increase the virucidal activity of metals, copper was mixed with peroxide before virus incubation. The data in Figure 3 indicate that an equimolecular mixture of copper and peroxide (Mix 1) is over twice as effective at inactivating viruses than is copper alone. Peroxide alone (Figure 3), just as in the experiment illustrated in Figure 1, exhibits relatively poor virucidal activity. A 1:1 mixture (Mix 1) at 6 mg/L inactivates approximately 50% of the original virus load, while the effect of copper and peroxide acting independently adds up to only about 24%.

The enhancement of inactivating activity of a low concentration of copper (0.05 mM, equal to 3.17 mg/L) was assessed in the presence of different proportions of peroxide. The data shown in Figure 4 clearly indicate that peroxide potentiates the virucidal capacity of copper throughout the range of ratios studied. Copper

alone at 3.17 mg/L was able to inactivate approximately 20% of infective virus particles. When 100 molecules of peroxide per copper molecule were present, 96% of the original virus load was inactivated after 30 minutes. Since the curve shown in Figure 4 does not reach a plateau at the bottom of the graph, it can be expected that even greater virucidal activity can be achieved at higher proportions of peroxide to copper.

D. Virucidal efficiency of a mixture of copper and peroxide

Figure 5 summarizes two independent experiments (see Table I) in which the virucidal activity of different concentrations of a mixture containing 100 parts of peroxide per part of copper (Mix 100) was compared with that of glutaraldehyde (Gl). The pH of the incubation media was 7.4, which is within the range for optimal activity of glutaraldehyde [Rubbo et al, J. Appl. Bact., 30:78 (1967)].

The ID_{50} for the mixture is reached at a copper concentration of 0.07 mg/L, while the ID_{50} for glutaraldehyde is not reached until 3.5 mg/L. This suggests that the mixture is approximately 50 times more efficient in inactivating viruses than glutaraldehyde when the concentration of copper and glutaraldehyde are compared.

E. Bactericidal effects

It was important to determine whether the high virucidal efficiency of the copper/peroxide mixture is paralleled by similar bactericidal behavior. The ability of a mixture of copper and peroxide to inactivate bacteria in the exponential growth phase was compared to the bactericidal activity of glutaraldehyde.

The survival data for E. coli shown in Figure 6 indicate that an ID_{50} is achieved at 0.45 mg/L of copper in the presence of 45 mg peroxide. In the same

experiment, 4.6 mg/L of glutaraldehyde were required to kill 50% of the bacteria after 30 min. incubation at room temperature. This result indicates that a mixture of copper and peroxide in a 1:100 ratio can kill bacteria more effectively than glutaraldehyde.

F. Kinetics of virus inactivation

The kinetics of virus inactivation of a mixture of 100 parts peroxide per part of copper as compared to that of glutaraldehyde was studied. The results of three independent experiments are shown in Figure 7. It is obvious from these data that the copper:peroxide mixture inactivates viruses much faster than does glutaraldehyde.

The inactivation rate shown in Table II was defined in an attempt to quantitate the speed with which different substances inactivate viruses. It is assumed as an approximation that the log of inactivation is linearly related to the incubation time and the concentration of active substance. This assumption is supported by the similar rates obtained for glutaraldehyde at 1 mg/L and 20 mg/L (Table II).

TABLE II

KINETICS OF VIRUS INACTIVATION

Concent.[a]	(% Serum)	50% Inactivation (min.)		Inactivating Rate[b] (% kill/min/mg/L)	
		Copper (100:1)	Glutar.	Copper (100:1)	Glutar.
1 mg/L	0.9	4	40	2.5	1.2
1 mg/L	5.0	7	50	7.1	1.0
1 mg/L	0.9	4	ND	12.5	---
20 mg/L	0.9	ND	3	---	0.8

a: Final concentration of either copper in the mixture 1:100 with peroxide, or glutaraldehyde (Glutar.), in which viruses were incubated (at room temperature).

b: Percentage of viruses inactivated per minute of contact with 1 mg/L of substance. Calculated by dividing the % of inactivation by the time necessary to inactive such amount, divided by the concentration of active substance in mg/L.

Glutaraldehyde exhibited an identical inactivating rate whether its source was a commercially available disinfectant (Cidex) or either a technical or reagent grade chemical. Identical performance was also obtained when Cidex was alkalinized by adding bicarbonate according to manufacturer instructions, or by raising the pH to 7.4 by diluting in buffered media.

The inactivation rate for infective virus particles incubated with a 1:100 mixture of copper and peroxide in the presence of 0.9% serum was about ten times faster than that of glutaraldehyde. In Eagle's minimal essential medium, pH 7.3, containing 5% serum, the copper mixture was still about seven times faster than glutaraldehyde in inactivating viruses.

The stability of the mixture was tested up to 22 hrs after mixing copper and peroxide. The inactivating rate remained constant throughout the 22 hr time span studied (data not shown).

G. Enhancement of virucidal potency by nitrilotriacetic acid (NTA)

The effect of nitrilotriacetic acid (NTA) at different concentrations on the virucidal activity of an iron/peroxide mixture is shown in Figure 8. A low starting virucidal activity was chosen to show the enhancing effect of NTA. A 1:1 mixture of iron and peroxide at a concentration four times below the ID_{50} (Table I) inactivated 6.5% of the viruses present. The addition of NTA in the range of about 0 to about 31 grams/L enhanced the inactivating potency of the system four to seven times. The same concentration of NTA alone did not inactivate viruses. The enhancing effect of NTA shown in Figure 8 was obtained in the presence of 0.9% fetal calf serum. This indicates that protein in the amount likely to be found in contaminated medical devices should not impair the potentiating effect of NTA. The enhancing effect of NTA was optimal at neutral or slightly alkaline pH; acid pH should be avoided. NTA performed best when it was mixed with the metal before treating the infectious sample. Metal chelators other than NTA can be employed.

Taken together, the data shown above indicate that glutaraldehyde is the most efficient virucidal substance in current use. However, a mixture of copper and peroxide is substantially better than glutaraldehyde. A mixture of copper and peroxide in a ratio of 1:100 is approximately 50 times more efficient in terms of grams of copper to grams of glutaraldehyde in achieving the same effect, and about 10 times faster in inactivating viruses. The addition of NTA to the metal/peroxide mixture increases even further the inactivating potency of the system.

DISCUSSION

The data presented supra demonstrate that copper and iron are capable of inactivating viruses. Considering

the amount required to inactivate similar loads of viruses and bacteria, these data indicate that metals such as copper and iron inactivate viruses with an efficiency five to ten times lower than that of glutaraldehyde, but five to forty times higher than that of recommended sterilizing substances such as formaldehyde, peroxide, or chlorine (Figs. 1 and 2; Table I).

In comparison to the concentration recommended for disinfection with peroxide and formaldehyde (6 to 10% and 8 to 12%, respectively), the results obtained via the present invention indicate that copper and iron at 0.2 to 2% can inactivate microorganisms with equal or better efficiency than other disinfectants currently in use.

The results described herein were obtained with copper (II) and iron (III). Metals in other oxidation states, such as copper (I) and iron (II), also show inactivating properties.

In spite of little previous data on its virucidal activity, peroxide remains a recommended sterilizing substance. The present data show that the virucidal activity of peroxide alone is relatively low (about 50 times less) when compared with glutaraldehyde.

The present invention discloses the discovery that peroxide potentiates to an unexpectedly high degree the virucidal and bactericidal effect of metals. This enhancement increases with the proportion of peroxide to metal in the mixture throughout the range tested. The enhancement by peroxide occurs in a concentration dependent fashion, and did not reach saturation in the range studied.

Copper or iron in a 1:1 mixture with peroxide has 60 to 70% the virucidal power of a similar amount of glutaraldehyde. Hence, a virucidal activity similar to that of 2% glutaraldehyde can be achieved with a mixture of 3% metal and 3% peroxide.

Increasing the proportion of peroxide:copper to 100:1 increases the virucidal power of the mixture above that of glutaraldehyde. Comparing the amount of copper and glutaraldehyde required to inactivate the same amount of viruses, a mixture of one part copper per 100 parts of peroxide is 50 times more efficient than glutaraldehyde (Fig. 5). A similar mixture of iron and peroxide is five times more efficient than glutaraldehyde.

The experiments performed via the tests described here indicate that a mixture containing 0.4 g copper and 40 g peroxide per liter (0.04% and 4%, respectively) should have similar virucidal properties to that of 2% alkaline glutaraldehyde.

Although the experiments performed herein were carried out up to a proportion of copper:peroxide of 1:100, it is likely that the virucidal activity of metals can be enhanced even further by increasing the concentration of peroxide.

The bactericidal properties of glutaraldehyde were compared to a mixture of copper and peroxide. The amount of copper required in a 1:100 mixture with peroxide was about ten times smaller than the amount of glutaraldehyde required to inactivate the same number of bacteria (Fig. 6).

The results presented in Fig. 7 indicate that the rate of virus inactivation by copper and peroxide (1:100) is faster than that of glutaraldehyde. The copper and peroxide mixture remains a more rapid inactivating agent than glutaraldehyde even in the presence of serum in the range tested (up to 5%, Table II). It is unlikely that more than 5% serum would be left after washing a device to be sterilized. In any case, if more than 5% serum is left in a medical device due to errors in the cleaning procedure, the choice of disinfectant becomes quite irrelevant.

While the virucidal data disclosed herein were obtained employing Junin virus, the compositions and

methods of the present invention are applicable to a wide variety of viruses. These include, for example, the viral agents which cause AIDS (HIV), rubella, equine encephalitis, yellow fever, dengue fever, the common cold, mumps, measles, influenza, hemorrhagic fever, variola, vaccinia, herpes, and hepatitis.

The compositions and methods of the present invention are also applicable to a wide range of bacteria and fungi, including, for example, Corynebacterium, Staphylococcus, Streptococcus, Pseudomonas, Gonococcus, Salmonella, Clostridium, Actinomyces, Nocardia, Candida, Blastomyces, Histoplasma, Coccidioides, and Sporotrichum.

The nature and/or use of a particular medical device may dictate that the levels of metal and peroxide be kept to a minimum. To address this situation, many substances were screened for enhancement of the virucidal potency of the metal-peroxide mixture. The results shown in Fig. 8 indicate that NTA can potentiate the virucidal activity of iron and peroxide over seven times.

The results obtained via the instant invention indicate that the addition of NTA allows reduction of the concentration of metal and peroxide in the mixture, while still retaining high virucidal potency. There is great flexibility in the proportion of the three elements in the mixture. An example of a formulation with microbicidal potency similar to that of 2% glutaraldehyde is given in Table III.

Based upon the results disclosed herein, metals, either alone, mixed with peroxide, or in a ternary system with NTA, can be advantageously used for the sterilization and disinfection of reusable medical devices such as bronchoscopes, surgical instruments, hemodialyzers, catheters, and others.

Comparison of the ID_{50} obtained in the present study, together with previous experience in the use of glutaraldehyde, suggests formulations with similar or higher virucidal potency than that of 2% glutaraldehyde.

Treatment with solutions formulated as described in Table III for 30 min. at room temperature should suffice to safely inactivate any viruses, including HIV, as well as bacterial spores.

The proportion of copper to peroxide can be optimized further for special applications. Elimination of peroxide altogether, while retaining similar microbicidal activity, can be employed if the metal concentration is increased accordingly. Addition of NTA or another chelator would allow reduction of the amount of both metal and peroxide, while maintaining the virucidal potency of the solution.

In normal human sera, the levels of copper and iron range from 1.1 to 2.3 mg/L [Fisher et al, Cancer, 37:356 (1976); Scientific Tables Geigy, p. 530 and 585 (1965)]. The amount of metal that could remain after washing medical devices sterilized or disinfected with either copper or iron in the concentrations reported here should be below the physiologic range. Hence, this should pose little or no health risk due to human exposure. This is an obvious advantage over current disinfection and sterilization methods.

Another advantage of this invention is the disclosure of sterilizing formulations of great flexibility. Depending upon the requirements and limitations of the situation, different sterilizing solutions can be employed by adjusting the concentrations of up to three compounds: metal, peroxide and NTA. The user can choose from a simple solution of aqueous copper, iron, or other metal salts, variable mixtures of metal(s) and peroxide, ternary mixtures composed of metal(s), peroxide, and NTA, or metal(s) and NTA. Several formulations with sterilizing activity similar or better than that of 2% glutaraldehyde (as established by comparing the ID_{50} and virucidal power described in Table II) are disclosed in Table III.

TABLE III

METAL-BASED FORMULATIONS WITH HIGH VIRUCIDAL POTENCY[a]

System		Concentration
Unitary		
Copper		10 % Cu(II)
Iron		20 % Fe(III)
Binary		
Copper:Peroxide	(1:1)	3.3 % Cu(II) and 3.3 % peroxide
	(1:100)	0.04 % Cu(II) and 4 % peroxide
Iron:Peroxide	(1:1)	2.9 % Fe(III) and 2.9 % peroxide
	(1:100)	0.4 % Fe(III) and 40 % peroxide
Tertiary		
		0.75 % Fe(III), 0.75 % peroxide, and 3.5 % NTA[b]

a: Formulations based upon the relative virucidal power shown in Table I.

b: Metal and NTA mixed together at pH > 7 before treatment.

Compositions of the present invention include those wherein the molecular ratio of transition metal to peroxide preferably ranges from about 1:1 to about 1:30,000, more preferably from about 1:1 to about 1:1,000, and most preferably from about 1:1 to about 1:200. The concentration of transition metal therein can preferably range from about 0.01 mg/L to about 10 kg/L, more preferably from about 0.1 g/L to about 50 g/L, and most preferably from about 0.4 g/L to about 10 g/L. The concentration of peroxide therein can preferably range from about 0.1 g/L to about 320 g/L, more preferably from about 2 g/L to about 50 g/L, and most preferably from about 3 g/L to about 30 g/L. Finally, the concentration of chelating agent in compositions of the present invention can be that which partially or totally chelates the metal therein, and can preferably range from about 0.01 mg/L to about 100 g/L, more preferably from about

100 mg/L to about 30 g/L, and most preferably from about 1 g/L to about 10 g/L.

The present findings regarding the high virucidal activity of metals alone, metals combined with peroxide, NTA, or ternary mixtures of all three, can prove beneficial in therapy. They offer an alternative for attenuation of microorganisms in vaccine development, and also allow for gentler treatment of medical grafts, prosthetic devices, and implants.

With respect to therapeutic uses of the compositions of the present invention, transition metals such as copper and iron are essential for human health, although they can be toxic in excess levels. The deleterious effects of excess copper intake have been shown to be reversible.

Industrial exposure to copper has not revealed any sign of chronic disease [Cohen, J. Occup. Med., 16:621-624 (1974]. Copper sulfate has been used clinically as an emetic in the treatment of intoxications [J. Aaseth and T. Norseth, in Handbook on The Toxicology of Metals, p. 244, Elsevier (1986)]. Treatment of more than 500 patients having rheumatoid arthritis with intravenous injections or infusions of 2.5 mg/day of copper complexed with salicylate did not produce deleterious effects [Hangarter, in Inflammatory Diseases and Copper, pp. 439-452, Humana Press (1982)].

The normal concentration of copper in whole blood is between 1 and 2 mg/L [Fisher et al, Cancer, 37:356 (1976); Scientific Tables Geigy p. 530 and 585 (1965)]. In erythrocytes, 60% of copper is bound to the enzyme superoxide dismutase; ceruloplasmin binds 95% of serum copper in humans [J. Aaseth and T. Norseth, in Handbook on The Toxicology of Metals, p. 241, Elsevier (1986)]. Under normal conditions, little copper is left free for any microbicidal action of the kind disclosed in this invention. Elevation of normal levels of transition

metals is therefore needed for virucidal or bactericidal therapeutic action.

The results shown in Table I indicate that 10 to 16 mg copper/L can inactivate 50% of the viruses present after 30 minutes exposure. The data in Figure 2 indicate that if 1 mg copper per liter is free to act as a microbicidal in the blood, about 25% of viruses would be destroyed after 30 minutes at 20°C, and even more at the body temperature of 37°C. These data indicate that elevation of the copper concentration just 1 mg/L over normal blood levels for an effective period, typically from about 3 to about 14 days, will have a therapeutic microbicidal effect.

The results obtained here, together with previous clinical and toxicological data on the administration of copper in humans, suggest that intravenous injection or infusion of about 1.5 to about 5 mg/day, preferably about 2 to about 4 mg/day, and most preferably about 2.5 to about 3.5 mg/day of copper can be therapeutically useful in the treatment of infectious diseases, particularly virosis. This regimen will elevate the copper concentration in the blood without reaching toxic levels. Furthermore, the simultaneous administration of copper, iron, or combinations of transition metals, with a metal chelator such as NTA, can increase the <u>in vivo</u> virucidal/bactericidal effect.

The invention being thus described, it will be obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

What is claimed is :

1. A disinfectant or sterilizing composition, comprising:

(a) an aqueous solution of a salt of a transition metal or metals, and
(b) a peroxide.

2. The composition of claim 1, further comprising a chelating agent.

3. The composition of claim 2, wherein said chelating agent is nitrilotriacetic acid.

4. The composition of claim 1, wherein the molecular ratio of said transition metal or metals to said peroxide is from about 1:1 to about 1:30,000, respectively.

5. The composition of claim 1, wherein the molecular ratio of said transition metal or metals to said peroxide is from about 1:1 to about 1 : 1,000.

6. The composition of claim 1, wherein the molecular ratio of said transition metal or metals to said peroxide is 1:200.

7. The composition of claim 6, wherein said transition metal is copper or iron.

8. The composition of claim 7, wherein the concentration of said copper is in the range of from about 0.1 mg/L to about 100 g/L.

9. A method for inactivating viruses or bacteria, comprising treating said viruses or bacteria with an

aqueous solution of a salt of a transition metal or metals.

10. The method of claim 9, wherein said transition metal is copper or iron.

11. The method of claim 9, further comprising including a peroxide in said aqueous solution.

12. The method of claim 11, further comprising including a chelating agent in said aqueous solution.

13. The method of claim 12, wherein said chelating agent is nitrilotriacetic acid.

14. A method for disinfecting or sterilizing laboratory equipment, laboratory bench surfaces, medical devices, tissue grafts, implants, prosthetic devices, or fluids, comprising treating said equipment, surfaces, medical devices, tissue grafts, implants, prosthetic devices, or fluids with an aqueous solution of a salt of a transition metal or metals.

15. The method of claim 14, wherein said transition metal is copper or iron.

16. The method of claim 15, further comprising including a peroxide in said aqueous solution.

17. The method of claim 16, further comprising including a chelating agent in said aqueous solution.

18. The method of claim 17, wherein said chelating agent is nitrilotriacetic acid.

19. A method for inactivating microorganisms for use in vaccine development, comprising treating said

microorganisms with an aqueous solution of a salt of a transition metal or metals.

20. The method of claim 19, wherein said transition metal is copper or iron.

21. The method of claim 20, further comprising including a peroxide in said aqueous solution.

22. The method of claim 21, further comprising including a chelating agent in said aqueous solution.

23. The method of claim 22, wherein said chelating agent is nitrilotriacetic acid.

24. A method for treating infectious disease in a mammal, including humans, comprising administering to said mammal an antimicrobial effective amount of a transition metal or metals, alone or in combination with a chelating agent.

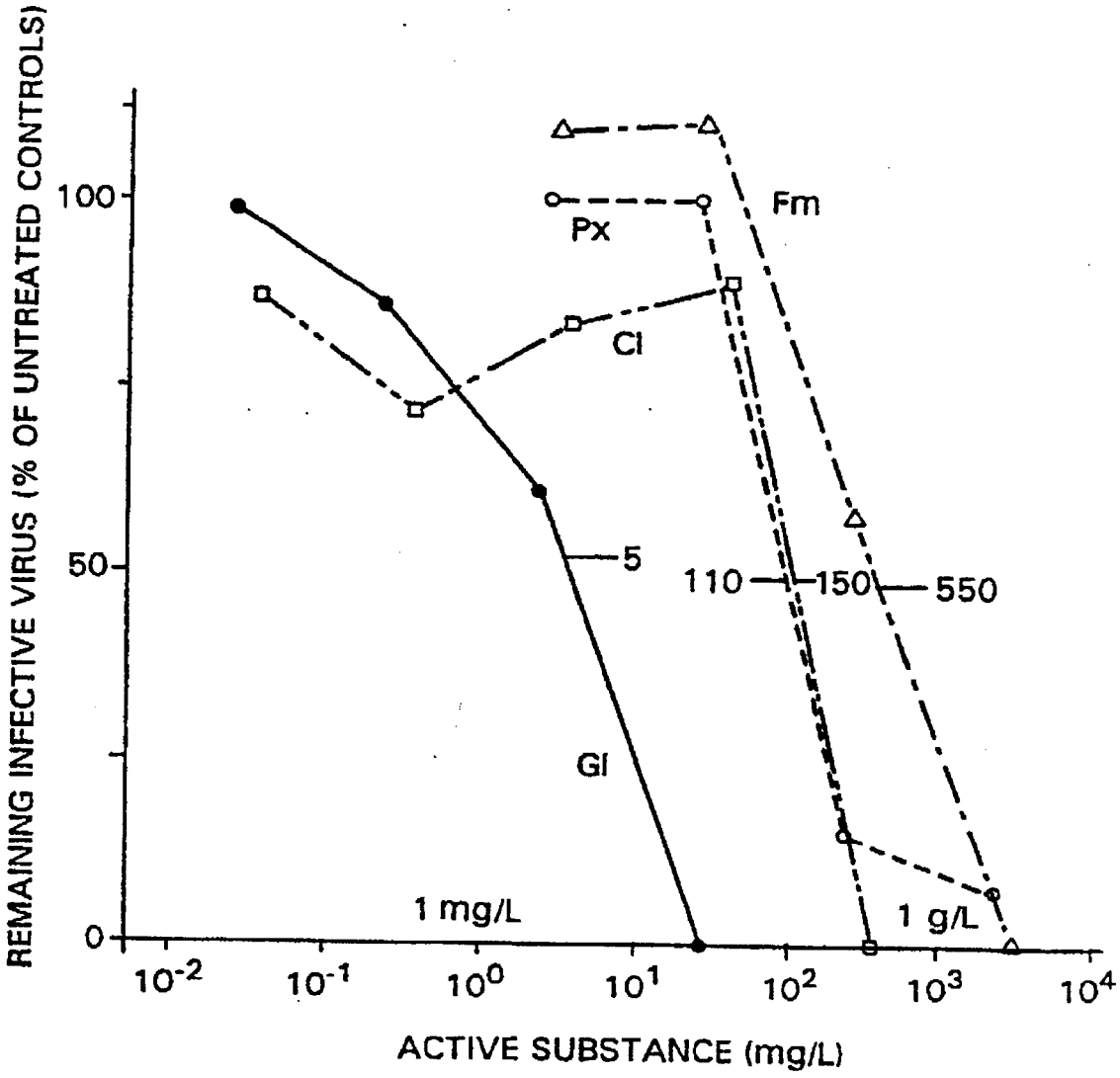
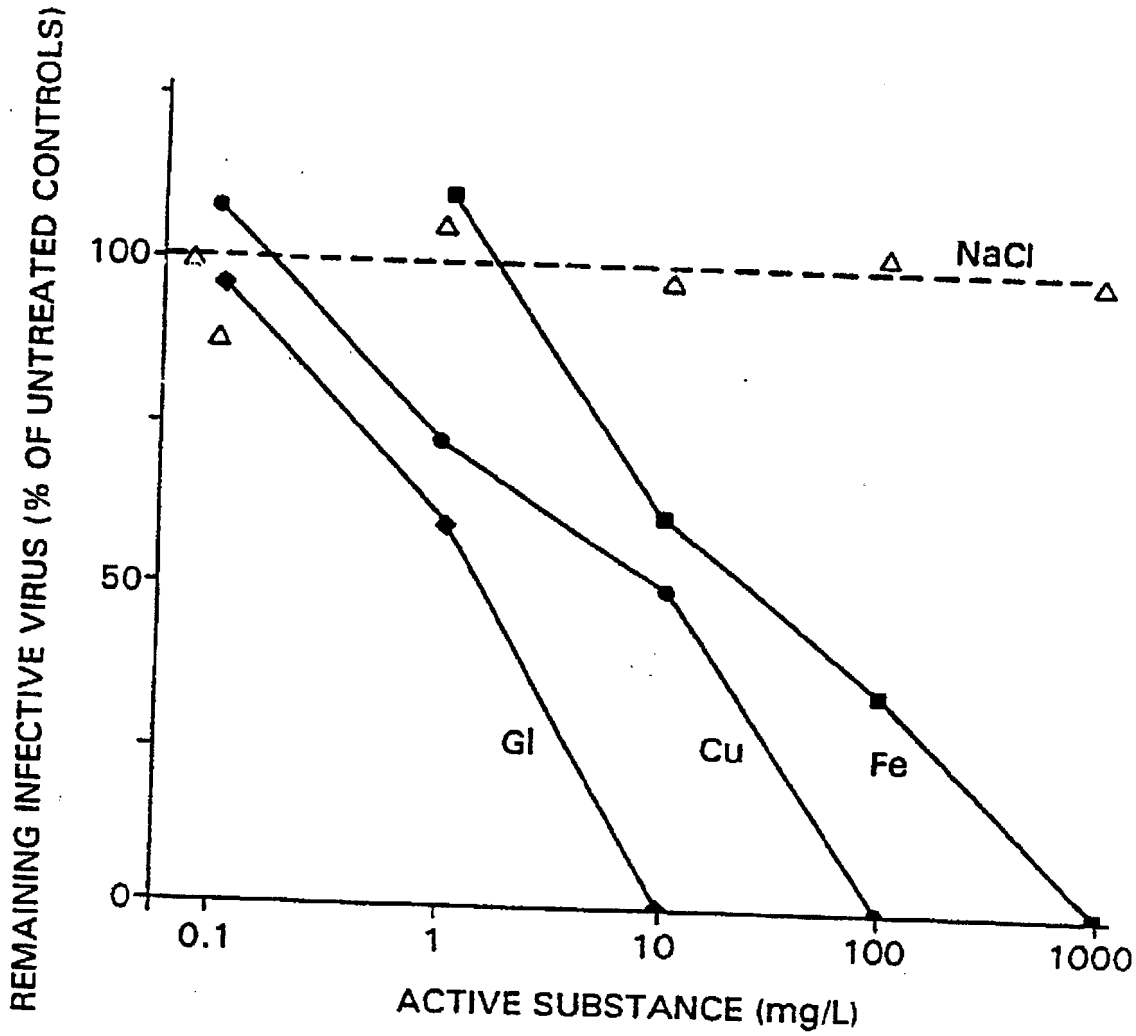

REMAINING INFECTIVE VIRUS (% OF UNTREATED CONTROLS)
100
50
0
Fm
Px
Cl
5
110
150
550
Gl
1 mg/L
1 g/L
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
ACTIVE SUBSTANCE (mg/L)

FIG. 1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NaCl
Gl
Cu
Fe
0.1
1
10
100
1000
ACTIVE SUBSTANCE (mg/L)

FIG. 2

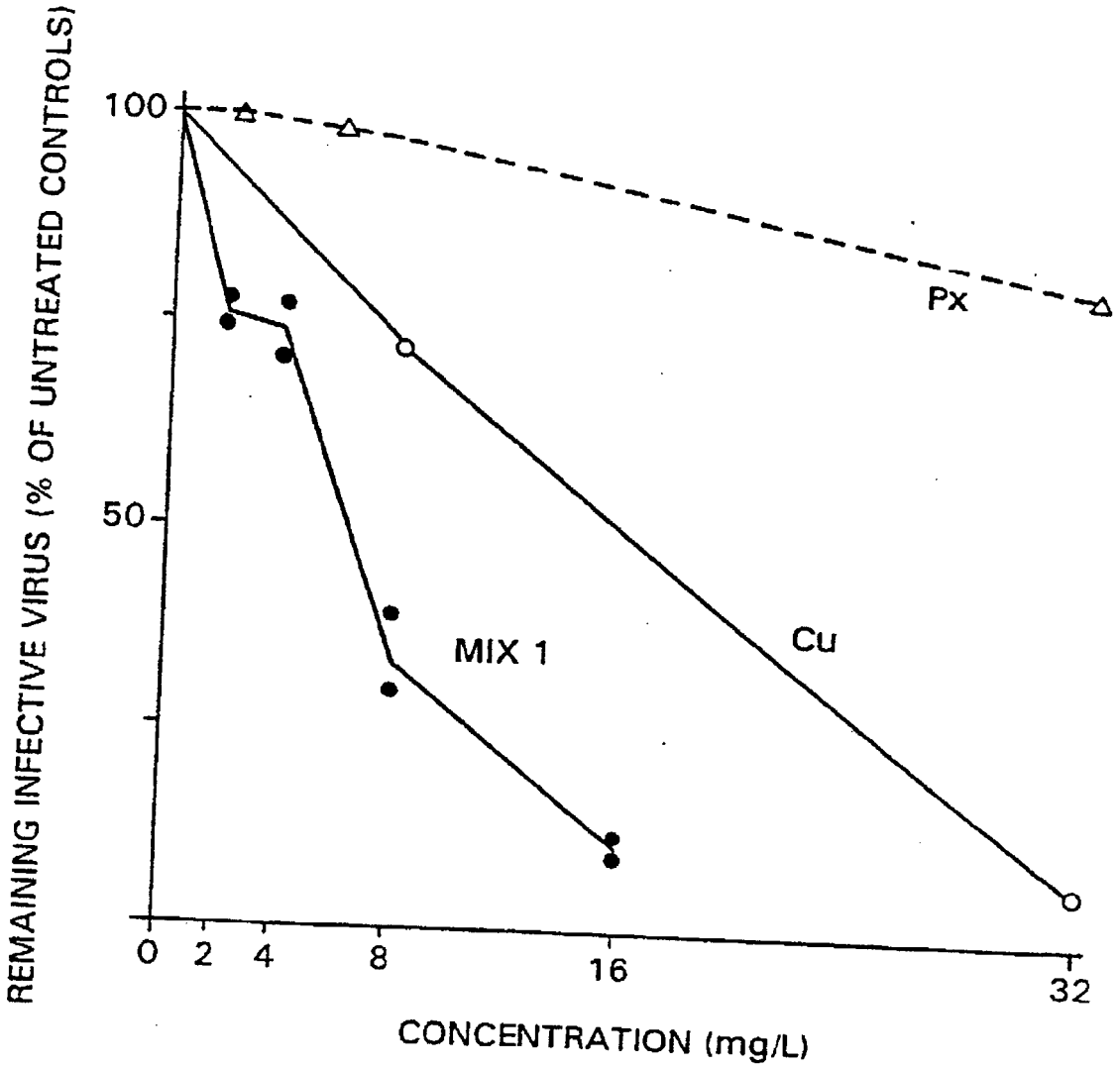
REMAINING INFECTIVE VIRUS (% OF UNTREATED CONTROLS)
100
50
Px
MIX 1
Cu
0
2
4
8
16
32
CONCENTRATION (mg/L)

FIG. 3

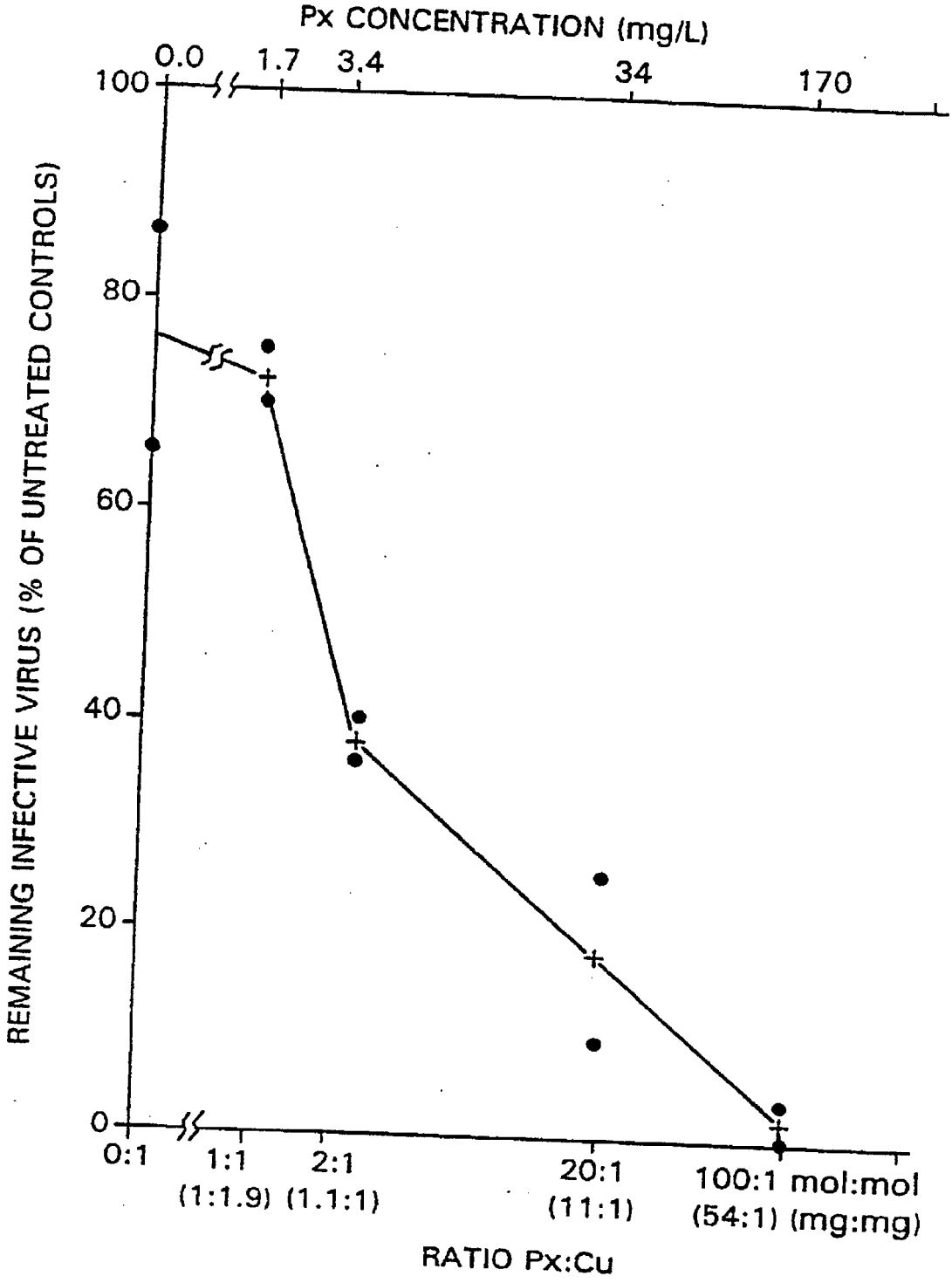
Px CONCENTRATION (mg/L)
0.0
1.7
3.4
34
170
100
80
60
40
20
0
REMAINING INFECTIVE VIRUS (% OF UNTREATED CONTROLS)
0:1
1:1
(1:1.9)
2:1
(1.1:1)
20:1
(11:1)
100:1 mol:mol
(54:1) (mg:mg)
RATIO Px:Cu

FIG. 4

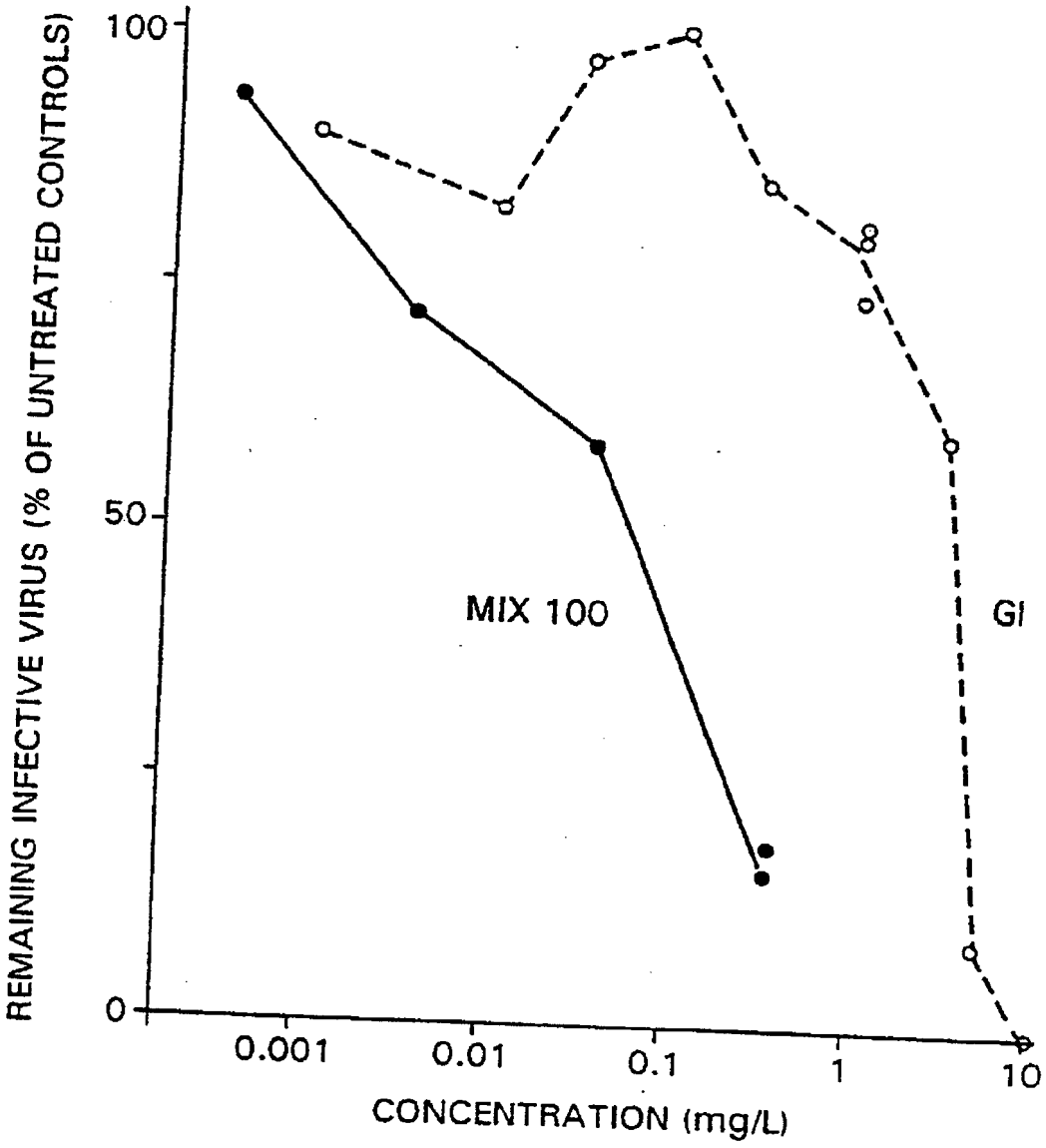

FIG. 5

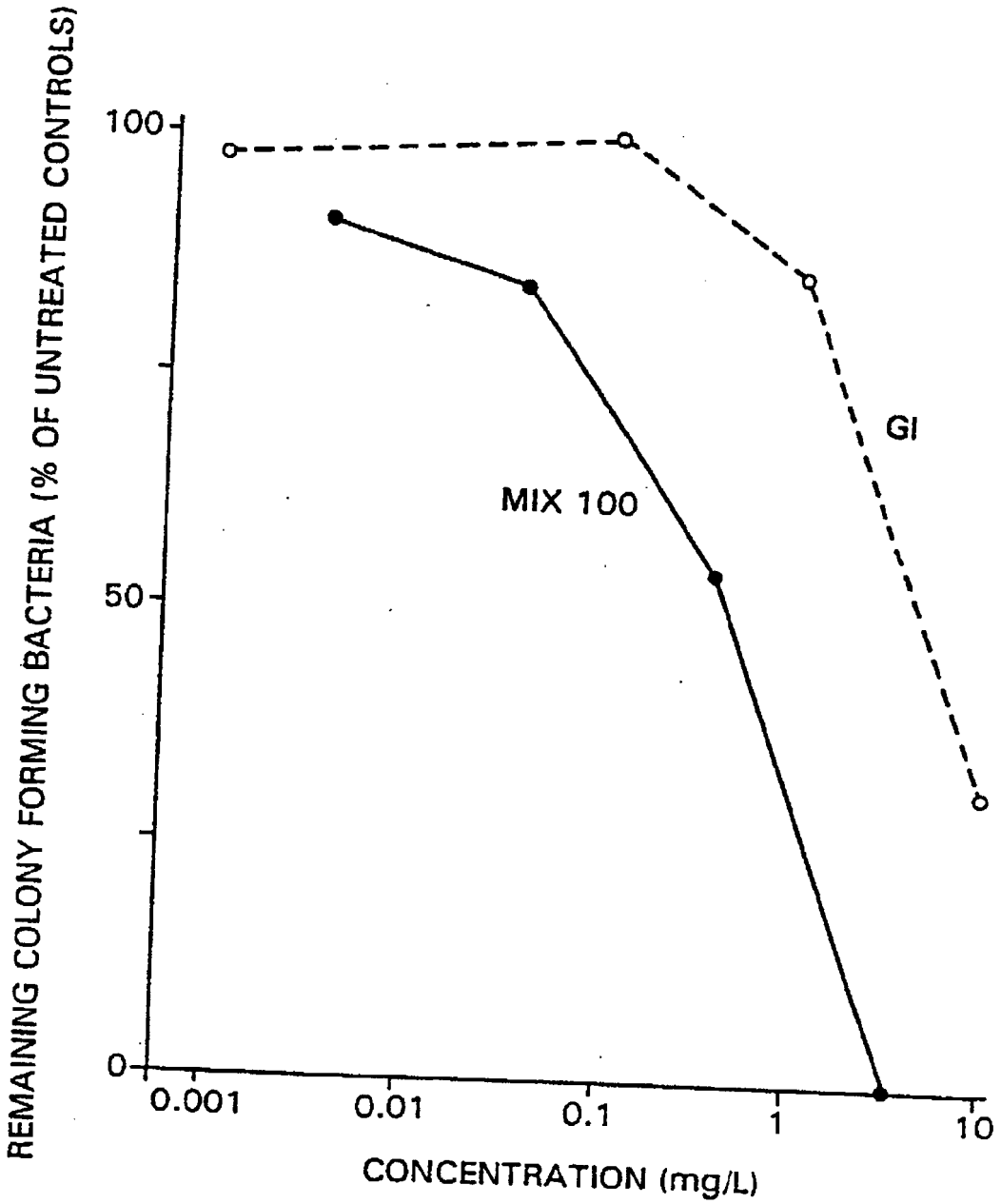
REMAINING COLONY FORMING BACTERIA (% OF UNTREATED CONTROLS)
100
50
0
0.001
0.01
0.1
1
10
CONCENTRATION (mg/L)
GI
MIX 100

FIG. 6

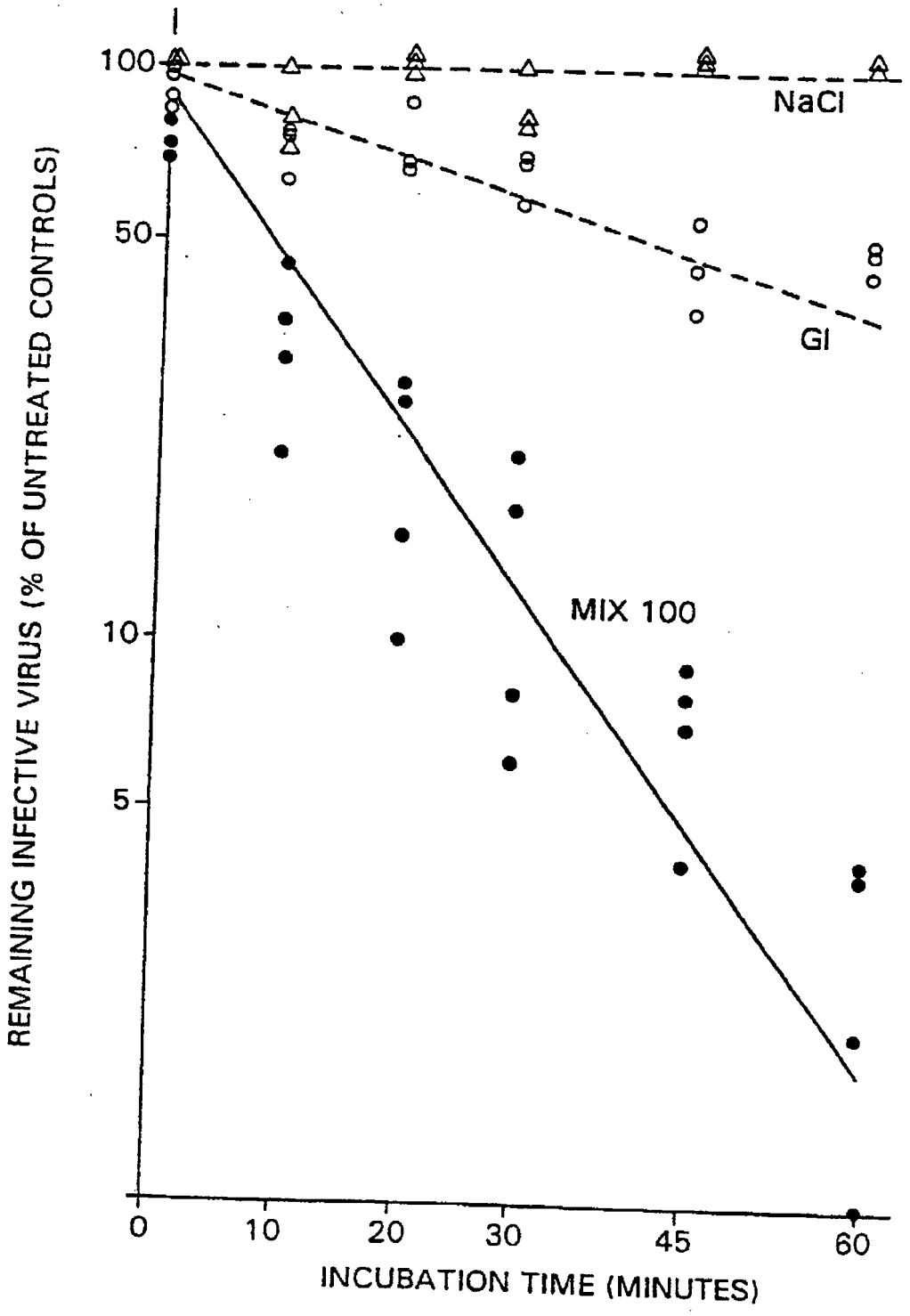

FIG. 7

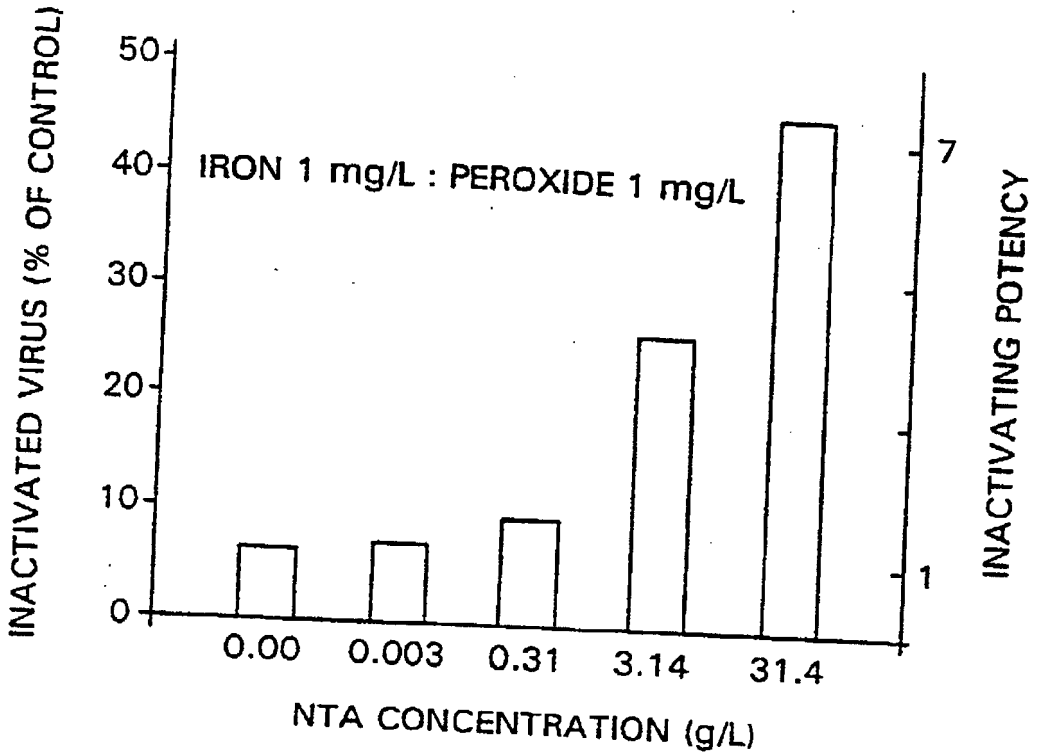
INACTIVATED VIRUS (% OF CONTROL)
50
40
30
20
10
0
IRON 1 mg/L : PEROXIDE 1 mg/L
7
1
INACTIVATING POTENCY
0.00
0.003
0.31
3.14
31.4
NTA CONCENTRATION (g/L)

FIG. 8

INTERNATIONAL SEARCH REPORT

PCT/US92/07238

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 33/40, 33/24, 33/26, 33/34; A01N 59/16, 59/20, 37/44

US CL :424/616, 617, 630, 633, 634, 635, 638, 646, 647, 648; 514/561

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/616, 617, 630, 633, 634, 635, 638, 646, 647, 648; 514/561

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,476,010 (Bouk, deceased) 09 October 1984, see, e.g. Examples I, VI and VII.	1,4-5, 9-11 14-16 1-24
X Y	US, A, 4,311,598 (Verachtert) 19 January 1982, see columns 1-2, table 1, and claim 1.	1,4-5, 9-11, 14-16 1-24
X Y	US, A, 4,822,595 (Corliss et al.) 18 April 1989, see column 4 and claim 1.	1 and 4-5 1-24
Y	US, A, 4,416,854 (Nielsen) 22 November 1983, see column 1 and claims 1-4.	1-24
Y	US, A, 4,677,143 (Laurin et al.) 30 June 1987, see columns 1-2.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 MARCH 1993	23 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JOHN PAK
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07238

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,521,375 (Houlsby) 04 June 1985, See column 1 and claims 1, 10.	1-24
Y	US, A, 4,119,557 (Postlethwaite) 10 October 1978, see columns 1-2 and claims 1-6	1-24
Y	US, A, 4,720,385 (Lembach) 19 January 1988, column 3, lines 48-53 and column 4, lines 36-42.	19-23
Y	The Merck Index, 10th ed. NJ, Merck & Co., Inc., 1983, pp. 697 and 944.	1-24
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Patents Index, Documentation Abstracts Journal Section Ch, Week 9147, 29 January 1992, Derwent Publications Ltd., London, GB; Class C, AN 91-346754	1-24

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS: copper, iron, cupric, cuperous, cuprous, ferric, ferrous, nitrilotriacetic acid, triglycine, biscarboxymethyl glycine, hydrogen peroxide, antimicrobial, antivir?, microbicid?, bactericid?, disinfect?, steriliz?, medical device#, develop (5A) vaccin?, weaken?, inactivat?, damag?. CA online: Reg. No. 139-13-9 & viricid? Virucid? antiviral and other similar antimicrobial synonyms.